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14. ABSTRACT: Oxidative stress may play a role in human oncogenesis, including breast cancer. The mitochondria are most common sources of reactive oxygen species (ROS) responsible for most oxidative stress. This project evaluates the role of mitochondrial abnormalities in oxidative stress in breast cancer development. Transgenic mice harboring mutant mitochondrial Complex II subunit targeted in the mammary glands will be characterized in terms of mitochondrial functions, ROS production and oncogenesis. The effects of oxidative stress in other transgenic mouse models of breast cancer or predisposed mice will be generated by cross-breeding and analyzed in terms of their courses of oncogenesis in the presence or absence of the mitochondrial mutant transgene, and hence oxidative stress. This study should provide significant information regarding the role of oxidative stress in breast cancer development and progression, and insights on whether antioxidants are beneficial in prevention and treatment of such important cancer in women.					
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INTRODUCTION

Oxidative stress has been postulated to contribute to numerous human diseases, including aging, neurodegeneration, cardiovascular disease, and cancer (1,2). Since most studies were conducted at epidemiological levels, the experimental proof for such a causative effect has been lacking. The overall goal of this project is to address this question in transgenic mouse modeling focusing on breast cancer.

Numerous mutations with various mitochondrial components have been demonstrated to cause increase in reactive oxygen species (ROS), the ultimate mediators of oxidative stress. In particular, mutations in the subunits of the mitochondrial Complex II have been demonstrated to produce structural abnormalities, impair energy production, electron transport, and generate ROS (3,5,9). To address the role of mitochondrial structural abnormalities and ROS in breast cancer development and progression, an advanced strategy has been implemented to over-express a subunit of the mitochondrial Complex II in the mammary glands of transgenic mice. The effects of such mitochondrial abnormalities and elevation of oxidative stress will be evaluated in these animals in terms of breast cancer development under normal and predisposed conditions. The first two years of this project have been devoted to testing the mutant mice in cell cultures. The results of the cell culture studies suggested that indeed cells expressing mutant Complex II subunits showed significant increases in oxidative stress, leading to apoptosis in short term. But when such mutant Complex II subunit was expressed for a long time, these cells had undergone mutational events, resulting in more aggressive growth. These findings are significant and have provided some insights on the evolutionary aspects of Complex II mutations. The methodology, i.e. cell culture system, does not provide a means to obtain specific cell clones stably expressing the mutant Complex II and still maintain the early effects of such mutation(s). An objective that is important to study the events leading to its/their contribution to oncogenesis. For the last year, we have redesigned our strategy for this project and have devoted our efforts in generating transgenic mice and establishing the transgenic mouse models of breast cancer.

BODY

Task 1. To construct and characterize mutant transgenic mice

Generation of *Lox-mSdhCV69E* and *Lox-mSdhDH102L* transgenic mice.

Initially, we generated *LoxP-mSdhCV69E* mice and *LoxP-mSdhDH102L* mice that were designed to express conditionally SdhCV69E or SdhDH102L mutant after Cre dependent recombination (Figure 1A, top panel) (4). CAG promoter is a ubiquitously active in most cell types, and is capable of directing strong expression of genes under its regulation. When both the Cre and the floxed mutant Complex II transgenes are in the same animals, the Cre transgene will be expressed in specific tissues (i.e. mammary cells) by the tissue-specific promoter. The Cre recombinase will then cleave off the the β geo gene (fusion gene of β -galactosidase and neomycin resistant gene) flanked by *loxP* sites, thereby repositioning the SdhCV69E/SdhDH102L mutant transgene under the direction of the strong CAG promoter, resulting in conditional expression of the transgene in the tissue, as specified by the tissue-specific promoter for the Cre-recombinase transgene (Figure 1A, lower panel).

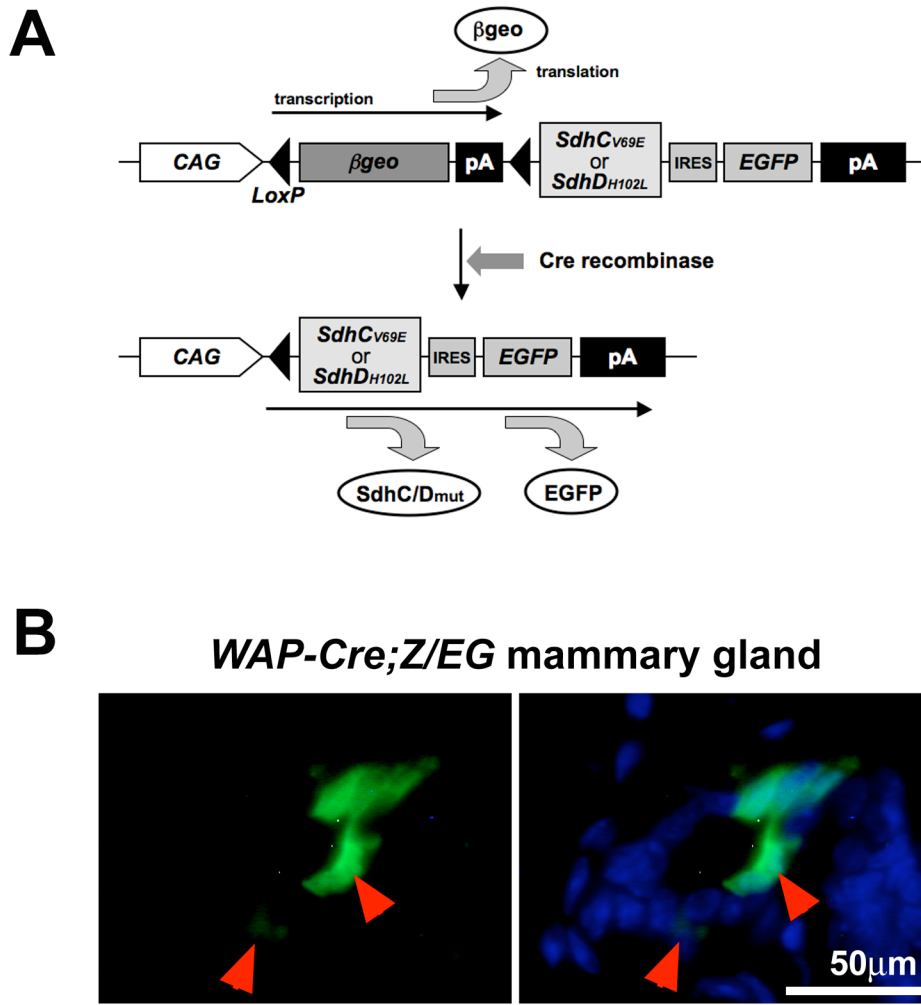


Figure 1. A) The diagrammatic illustration of Cre/LoxP transgene activation system. *LoxP-SdhC^{V69E}/DH102L* transgenes consists of two expression cassettes, β geo and *SdhC^{V69E}/DH102L-IRES-EGFP*, directed by CAG promoter. The β geo gene is normally expressed in transgenic mice. In double transgenic mice harboring both *LoxP-SdhC^{V69E}/DH102L* and Cre expressing transgenes, Cre recombinase cleaves the β geo gene flanked by *LoxP* sites, thereby *SdhC^{V69E}/DH102L* and *EGFP* genes are expressed under the control of CAG promoter. B) Left, image of EGFP (green fluorescence) expression in mammary gland of *WAP-Cre;Z/EG* double transgenic mouse. *Z/EG* mouse harbors the reporter transgene expressing β geo before recombination, and expresses EGFP after Cre-dependent recombination. Thus the EGFP expression corresponds to tissue-specific (i.e. mammary) activity of the WAP promoter in *WAP-Cre;Z/EG* double transgenic mouse. Right panel shows a merged image of EGFP (green) and DAPI staining for DNA (blue).

Using pronuclear embryo microinjection method, we have generated 13 (3 male and 10 female mice) and 7 (7 female mice) founder animals for *LoxP-mSdhC^{V69E}* and *LoxP-mSdhDH102L*, respectively.

Analysis of recombination of *Lox-mSdhCV69E* mice.

Five animals out of 13 founder animals of *Lox-mSdhCV69E* represent β -galactosidase activity in tissues, indicating that the transgene was active in these animals. Three lines (lines 15, 45 and 56) were established from these 5 founders. These lines were mated with Cre-recombinase expressing mice, e.g. *TSPY-Cre* mouse (7) and *WAP-Cre* mouse (8), to stimulate recombination in the target tissues. *TSPY-Cre* mouse expresses Cre-recombinase in germ cells and neurons. *WAP* (Whey Acidic Protein) gene promoter is specifically active in alveolar epithelial cells of mammary tissue during lactation (9), and therefore the *WAP-Cre* mouse expresses Cre in alveolar epithelial cells of mammary tissue (Figure 1B). Although we were able to observe selected cells in the mammary gland of double-transgenic mice expressing the reporter EGFP, we were not able to observe extensive expression of the Complex II mutant in double transgenic mice harboring both the *Lox-mSdhCV69E* and *WAP-Cre* transgenes, so far. These problems could potentially be attributed to the integration sites of the transgenes, the effectiveness of the Cre recombinase in cleaving the *loxP* sequences. Indeed, recently, others have reported that pronuclear injection might result in multiple-copy integration of the responder (*loxP*-tagged) transgene. Multiple-copy integration may cause undesirable number of *loxP* sites and possible chromosomal instability (10). We surmise that it might be the cases for the *LoxP-mSdhCV69E* transgenic mice, thereby resulting in efficient activation of the Complex II mutant transgene in the double transgenic mice, observed in our studies.

Analysis of abnormal observation with *LoxP-DH102L* mice.

Only one line of *LoxP-mSdhDH102L* (line 5, established from founder ID#5) represented the β -galactosidase activity in tissues. This line also could not be stimulated recombination in double transgenic mice with Cre-expressing lines. However, unexpectedly, one female mouse (15 month old) formed tumor in vagina and uterine cervix without Cre dependent recombination (Figure 2A and 2B). Some metastatic tumors were also observed in kidney (Figure 2C) and liver in this animal (Figure 2D). In addition, another founder female animal (ID#6, 17 month old) formed tumor-like structure in left lung (Figure 3A and 3C). In this animal, the transgene seemed to be active in mosaic manner, and only tumor-like cells of lung showed β -galactosidase activity (Figure 3E, arrows). Since the CAG promoter is extremely strong promoter and the β geo gene was flanked by possible alternative-splicing sites, we surmised that leaky expression of *SdhDH102L* might have caused abnormal cell growth without Cre-mediated recombination. These preliminary results suggest that this Complex II mutation could potentially exert oncogenic effects on its host animals. Unfortunately, we were not able to observe any tissue-specific, i.e. mammary cells, activation of the transgene after crossing with the *WAP-Cre* transgenic line. Based on these findings, we have re-designed our strategy, as discussed in the previous report, and initiated additional studies to generate transgenic mice harboring a mammary cell specific *mSdhDH102L* transgene.

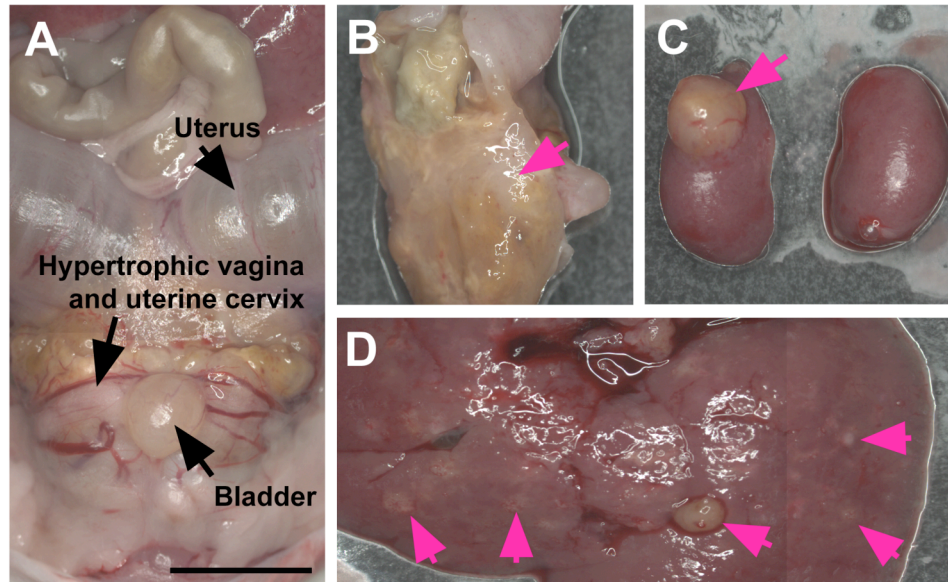


Figure 2. Tumor formations in a female *LoxP-mSdhDH102L* mouse (line# 5, 15 month). **A)** Intraperitoneal image showing uterus, uterine cervix and vagina. Uterine cervix and vagina were abnormally hypertrophied, and uterus was filled with fluid. **B)** Cross section of vagina and uterine cervix. **C)** Metastatic tumor formed in kidney. **D)** Morphology of liver showing abundant white dots were observed in (arrows). Scale bar = 10mm in A-C, 83mm in D.

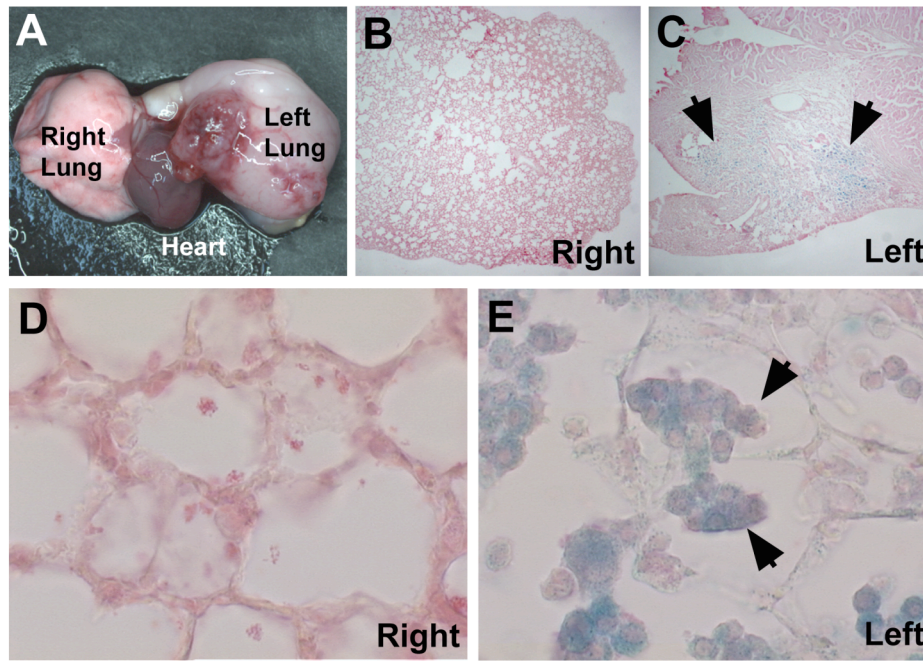


Figure 3. Hypertrophic lung in a female *LoxP-mSdhDH102L* mouse (ID#6, 15 month). **A)** Image of lung and heart. The left lung was bigger and whiter than the right lung. **B)** and **C)** X-gal stained sections of right lung (**B)** and left lung (**C)** at low magnification. β -galactosidase positive cells were stained blue (arrows). Sections were counter stained by Nuclear Fast Red to visualize nucleus (red color). **D)** and **E)** High resolution images of **B)** and **C)**, respectively. Blue cells, β -galactosidase positive cells, were filling the pulmonary alveolus in left lung (arrows in **E)**). Scale bar = 100 μ m in **D)** and **E)**.

Generation of WAP- *DH102L-V5tag/EGFP* mice.

Since we encountered several difficulties in the transgene activation in our double-transgenic mice with the Cre/loxP system, we have decided to directly express the mutant mSdhDH102L Complex II subunit in the mammary glands of transgenic mice directed by the WAP promoter. To establish these lines, we have performed further microinjections and generated the WAP-*msdhDH102L-V5tag/EGFP* mice to study the effects of this mutant on mammary gland (Figure 4). This mutation is selected because we observed numerous tumorigenic events among the hosts of the *LoxP-mSdhDH102L* transgene, as described above. The WAP gene promoter is specifically active in alveolar epithelial cells of mammary tissue (ref 9 and Fig. 1B), thereby should be able to direct the mutant transgene expression in the mammary target cells. We have also incorporated the epitope V5 as a tag for the SdhDH102L mutant, thereby allowing a more efficient means to detect the expression of this mutant transgene. Again, we have also included the fluorescent biological marker, EGFP, for convenient in vivo imaging of the tissue-specific transgene expression. Our efforts, so far, have produced 11 male and 11 female founder animals harboring transgene. Currently, these founder animals are mated with non-transgenic mice to form transgenic lines and to analyze transgene expression in mammary gland.

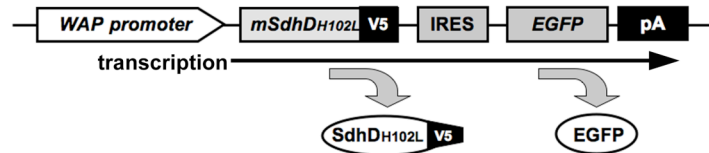


Figure 4. Structure and organization of WAP-*SdhDH102L-V5tag/EGFP* transgene. The expression of *SdhDH102L* and *EGFP* is controlled by WAP promoter that is specifically active in alveolar epithelial cells of mammary tissue. The IRES sequence between *SdhDH102L* and *EGFP* genes provides a bicistronic translation of V5-tagged SdhDH102L and EGFP. V5 is incorporated as a tag for SdhDH102L expression.

FUTURE DIRECTIONS

We obtained preliminary results in the transgenic mice harboring a mutant Complex II subunit, mSdhD-H102L, suggesting that this mutation might be involved in tissue vascularization and/or oncogenesis in host animals. Hence, we plan to focus on this particular mutation in our studies in the coming year. To expedite our investigation, we have refined our transgenic mouse strategy to a more simplified one, targeting the expression of the mutant Complex II by mammary specific promoter in the mammary glands of the transgenic mice. This improved approach allows a more ready evaluation on the effects of mutant subunit in mammary oncogenesis. However, we believe that the Cre-LoxP gene activation system still a significant strategy in the future, and probably might need to be used beyond the one-year period remaining in this project. We have now generated a total of 11 founding animals harboring a mammary specific mutant SdhDH102L. We have now requested a 12-month no-cost extension of this project and plan to finish the analyses of these transgenic lines in this coming year. The establishment of a breast cancer model involving the expression of a mutant Complex II subunit is a significant step towards the evaluation of mitochondrial structural abnormalities and dysfunction and oxidative stress in this human disease.

KEY RESEARCH ACCOMPLISHMENTS

- Construction of transgenic mouse lines harboring mutant Complex II mutant expression cassette
- Analysis of mammary gland-specific Cre recombinase and Complex II mutant double transgenic mice
- Characterization of founder animals harboring mutant Complex II mSdhD-H102L transgene, demonstrating the potential involvement of this mutant gene in tissue vascularization and oncogenic lesions
- Refinement and simplification of the transgenic strategy to demonstrate the significance of the mSdhD-H102L mutation in mammary oncogenesis
- Successful generation of transgenic founders harboring a mammary gland-specific SdhDH102L transgene

REPORTING OUTCOMES

None.

CONCLUSION

We have made good progress in evaluating the Cre-loxP strategy for tissue-specific transgene activation for the studies proposed in the original application. We have demonstrated the potential significance of a mutant Complex II subunit, mSdhD-H102L, in tissue vascularization and oncogenesis. We have now simplified our transgenic approach specifically designed to obtain some useful results, by directly targeting the SdhDH102L mutant in mammary glands of transgenic mice. We believe studies of these transgenic mice should provided significant insights on the role of mitochondrial abnormalities and dysfunction in oxidative stress and mammary oncogenesis.

SO WHAT

Oxidative stress has been implicated in the etiologies of numerous human diseases. Successful implementation of the proposed research will provide critical insights on its role(s) in breast cancer. The availability of experimental animal models of breast cancer, pertaining to mitochondrial structural abnormalities and oxidative stress (11), will be important in understanding the disease mechanisms, potential prevention and therapeutic intervention for this devastating human cancer.

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